Nitrate Measurement with Biosensor Technology

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Abstract

Protecting the water quality of rivers and watersheds will require improved methods for detection and analysis of compounds of environmental concern. The new methods will need to be economical and easy to use, so that the frequency of data collection and analysis can be increased. These goals need to be reached without compromising on accuracy, so that the data can be trusted and used. The new methods should also be developed with an emphasis on use of non-hazardous and nontoxic reagents, to keep both the user and the site uncontaminated and safe.

Bioanalytical methods, including immunoassays and enzyme-based assays, are beginning to bring these qualities and capabilities to quantitative analysis. Bioanalytical methods can provide data of accuracy and specificity comparable to instrumentation-driven analyses at far less cost, with the added advantage of being environmentally benign. Many of these methods can provide quality data both in the laboratory and on site in the field.

Enzymes are the protein catalysts that speed the chemical reactions that make living systems run. Enzymatic reactions are fast, efficient, very specific, and require little energy. Nitrate reductase is the enzyme that begins the process by which plants make their proteins. Ironically, it is also one of the physiological reasons that nitrate fertilizers are so effective in increasing crop productivity. These qualities make nitrate reductase a powerful tool for nitrate analysis.

Our company is dedicated to the application of enzymes to the solution of environmental problems. Our first products are a series of nitrate test kits based on the enzyme nitrate reductase, which we purify from corn seedlings. Kits based on our enzyme have been in use for biomedical research since 1995. Funding from the USDA's Small Business Innovation Research program has helped us to develop a series of nitrate test kits for analysis of nitrate in water. We are also involved in the development of a true nitrate biosensor, an electronic device capable of nitrate determination without the need for chemistry.

These kits use a chemistry similar to many other nitrate test kits, except that the heavy metal catalyst they require, cadmium or zinc, is replaced by nitrate reductase enzyme driven by the biological electron donor NADH (nicotine adenine dinucleotide, reduced form). The catalytic rate of NaR is about 200 nitrate to nitrite conversions per second per molecule of NaR. The reaction is irreversible and goes to completion:

$$\mathbf{NADH + NITRATE \rightarrow NITRITE + NAD^+ + OH^-}$$

A series of kits for a variety of applications has been developed. Lab kits provide quantitative data, comparable to Ion Chromatography or Ion Autoanalyzers, at a fraction of the cost, and have been formulated in test tube and 96-well microtiter plate formats. Field kits for on site or classroom analysis are quantitative when the test results are read with a colorimeter, and semi-quantitative data when a color chart is used. The Consumer kit allows anyone to test for nitrate at home, on site, or on the farm, even when the sample is dirty or colored. See Figures for Standard curves generated by enzyme-based nitrate analysis in freshwater and in seawater.

The next step in enzyme technology is the biosensor. The chemistry of the enzymatic reduction of nitrate involves a flow of electrons from a biological electron donor - such as NADH - through the enzyme and to the nitrate, reducing it to nitrite. In a biosensor, the enzyme is immobilized onto a solid support material, and electrons are supplied either directly by an electrode, or via an electron-carrying molecule attached to the same support. As nitrate is reduced, there is a tiny flow of electrons, a current, through the enzyme, and this current can be amplified and detected. The amount of current (in nano- or microamps) correlates to the concentration of nitrate present in the medium in which the biosensor probe is in contact. Data generated by our nitrate biosensor is shown below.

Nitrate biosensor technology is still in the prototype stage, but one key barrier has been the need for a purified and stable enzyme preparation; this obstacle has been overcome by our enzyme purification technology. Current efforts toward development of a practical device involve examination of enzyme immobilization techniques, how to eliminate oxygen interference from the system, and optimal biosensor materials. The goal is to develop a device whereby nitrate concentration can be read on a meter as the nitrate sensing probe is immersed in the sample.

Summary:

In order for increased nutrient analysis to become mandated, methods will need to be easy and also inexpensive. The methods also need to be accurate, or the data will have little meaning. Enzyme-based assays may be part of the solution to better protection of our water resources. Enzyme-based nitrate testing has significant advantages over competing methods. Because enzymatic reactions are specific, this method can be used in a wide variety of difficult samples. Highly colored and particulate samples are not a problem because these interferences are diluted out in the assay. Nitrate reductase-based nitrate testing is versatile: samples types investigated to date include biological fluids, seawater, and maple syrup.

Contamination of the environment by excess nitrate is a growing problem world-wide. Better nitrate monitoring capabilities may help solve the problem. An accurate, sensitive and safe nitrate measurement method can be a useful tool for regulators, agribusiness, and citizens to monitor how nitrate moves in the environment.

References:

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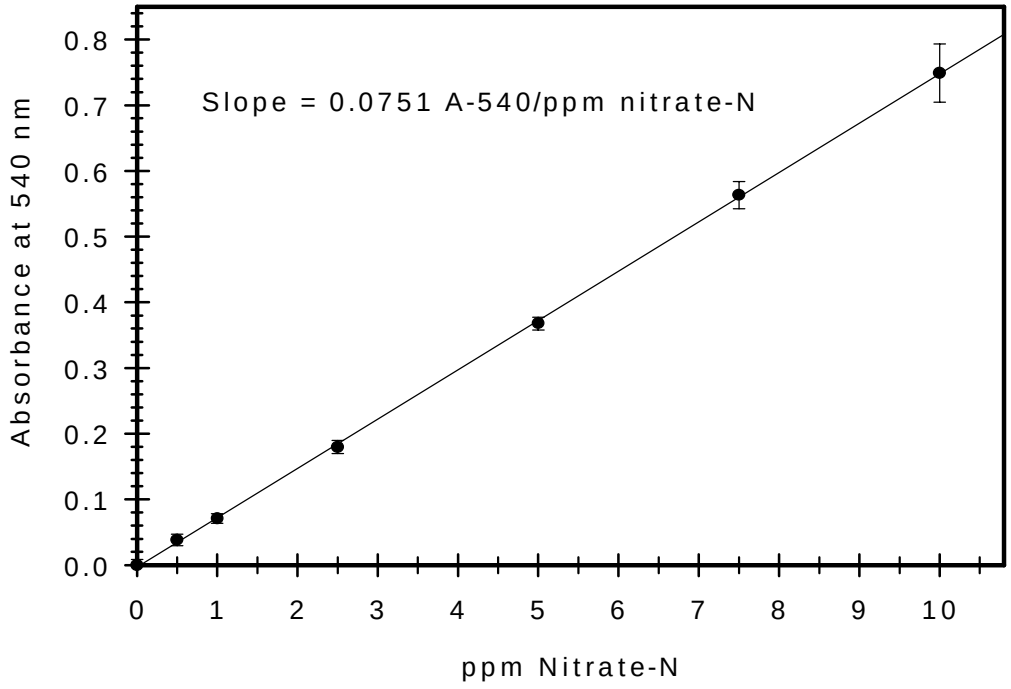
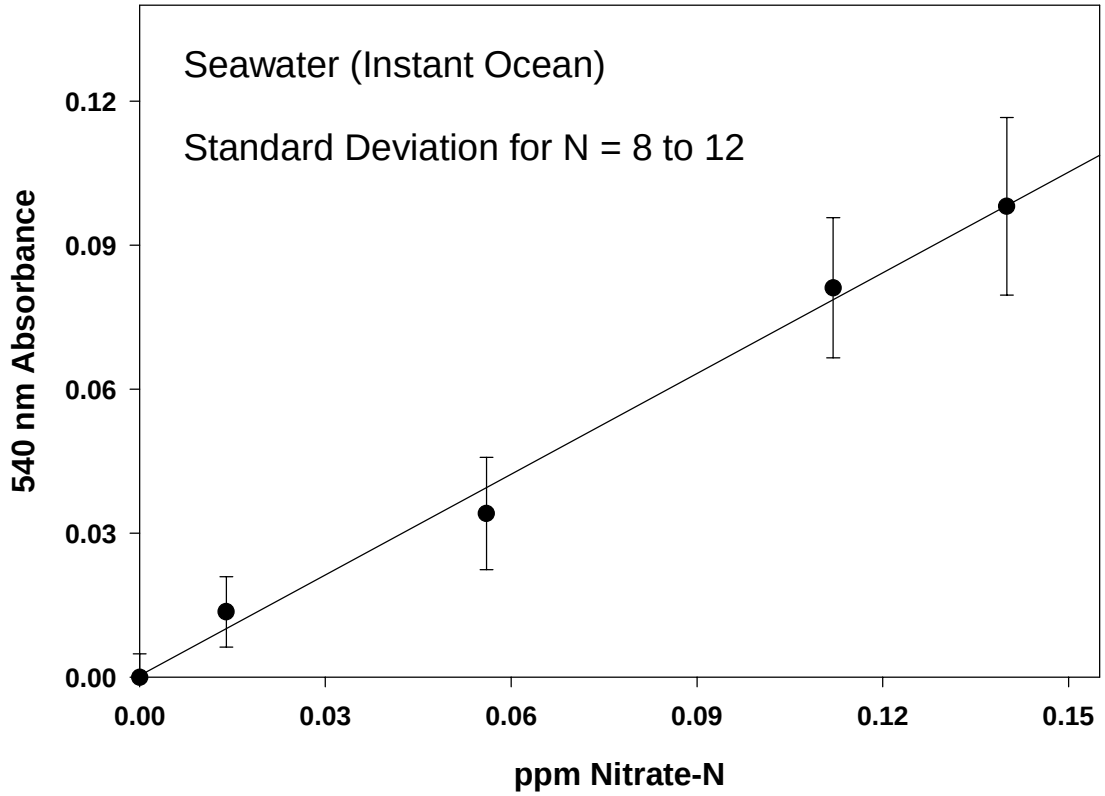


Figure 1. Typical Standard Curve generated with enzyme-based nitrate analysis. For nitrate analysis in this range, 50 µl of sample is added to one milliliter of reaction mix containing nitrate reductase (5 µl sample in 100 µl for the microwell kit), NADH, and buffer. After a reaction time of 15 minutes, at which time all (>95%) of the nitrate present in the sample will have been reduced to nitrite, 1 ml (100 µl in the microwell assay) of the Griess color reagents - acidified sulfanilamide and 0.02% N-Naphthylethylenediamine - are added. The resulting pink color is read using a spectrophotometer at 540nm (± 20nm), or by eye versus the standards and color chart provided with the Field and Consumer kits. The low sample volume in relation to total assay volume eliminates virtually all problems caused by color, pH extreme, or other contaminants in the sample.

Figure 2. Low level nitrate analysis in Seawater (Instant Ocean). Note that 0.15 ppm Nitrate-N (approx. 0.7 ppm nitrate) is equivalent to 10.7 µM Nitrate. This level of sensitivity in the presence of NaCl requires a larger sample volume of 500 µl and must be



read using a colorimeter or spectrophotometer. Low Range kits for analysis of nitrate between 0.025 - 1.0 ppm nitrate-N can be used with either the color charts or a colorimeter. The only interference found to date is permanganate or other strong oxidizers in the sample, which destroy the NADH; there are then no electrons available to the enzyme for nitrate reduction. Again, this is only an issue for the Low Range kits where the sample size is a larger portion of the total reaction volume.

Performance Characteristics of the NECi NaR-Nitrate Biosensor. When the NaR-NBS configuration shown in Fig. 1 is calibrated with anaerobic nitrate standards, a saturating current response is observed as shown in Table 1 and Fig. 3. Steady state current is obtained in 1 min after introducing a new nitrate aliquot.

Table 1. Current Response to Nitrate in the NaR-Nitrate Electrode

Normalized Current Response	Nitrate Conc (µM)	Nitrate-N Conc (ppm Nitrate-N)
0.00	0	0.00
0.01	3	0.04
0.05	10	0.14
0.11	25	0.35
0.35	100	1.40
0.60	250	3.50
0.81	500	7.00
0.97	1000	14.0
1.00	1200	16.8

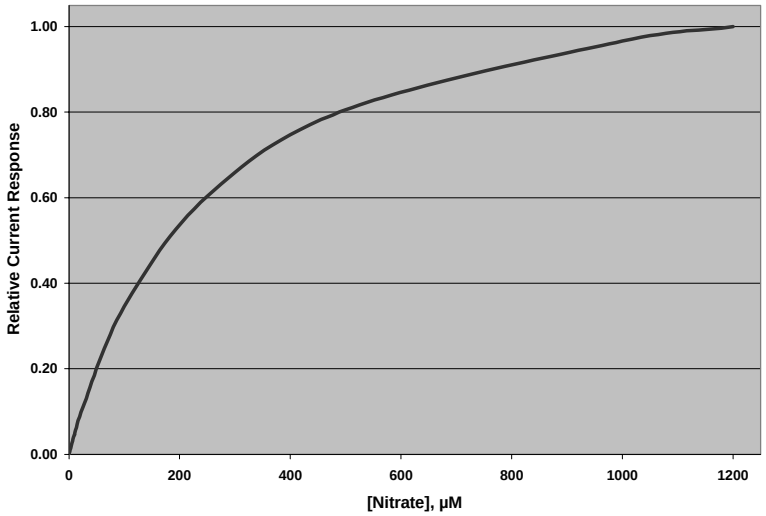


Figure 3. Current Response Curve for Calibration of the NaR-Nitrate Biosensor. Data from Glazier, Campbell & Campbell (1998).